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\textbf{In vivo} quantitative Raman-pH sensor of arterial blood based on laser trapping of erythrocytes

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We report on a continuous and non-invasive approach \textit{in vivo} to monitor arterial blood pH based on laser trapping and Raman detection of single live erythrocytes. A home-built confocal laser tweezers Raman system (LTRS) is applied to trace the live erythrocytes under different pH values of extracellular environment to record their corresponding Raman changes \textit{in vitro} and \textit{in vivo}. The analysis results \textit{in vitro} show that when the extracellular environment pH changes from 6.5 to 9.0, two Raman intensity ratio ($R_{1603,1616}$) of single erythrocytes decreases regularly, what is more, there has a good linear relationship between these two variables, and the linearity is 0.985, which is also verified successfully \textit{in vivo} Raman measurements. These results demonstrate that the Raman signal of single live erythrocytes is possible as a marker of extracellular pH value. \textit{In vivo} and quantitative Raman-pH sensor of arterial blood will be an important candidate for monitoring the acid-base status during treatment of ill patients and some major surgery because of its continuous and non-invasive characters.

1. Introduction

Continuous arterial blood pH monitoring is very desirable in clinical practice. For example, the measurement of arterial blood pH is one of the most frequently ordered tests in a critical care unit and in the process of some major surgeries.\textsuperscript{1,2} There have been lots of efforts on developing continuous arterial blood pH monitoring system using different types of sensors,\textsuperscript{3-6} such as metal/metal oxide electrodes, fiber-optic sensors, etc. However, these instruments have not been widely used in clinical practice due to invasiveness and high cost. To date, the measurement of arterial blood pH is mainly based on conventional blood gas analyzer and its limitations are as follows: First, it is cost-time and cannot depict the physiologic events continuously, which may cause the delay of therapeutic response; Second, it needs blood sampling from patients, which is invasive and may cause physical injuries and mental trauma to patients. Therefore, a continuous and non-invasive method of measuring blood pH is needed to solve these problems in clinical practice.

Erythrocytes, the highest number cells in blood, the function of that is directly influenced by temperature, osmotic pressure and pH of extracellular environment.\textsuperscript{7,8} In the body, blood is the external environment of erythrocytes, so the normal pH value for erythrocytes is 7.35-7.45.\textsuperscript{9} If the pH value goes beyond this range, the erythrocytes in blood are deformed with the hemoglobin structure changing to loss the function of transportation of oxygen and carbon dioxide.\textsuperscript{10} There have been lots of reports on studying morphological and molecular changes of the erythrocytes under different pH environments by means of physical and chemical methods respectively.\textsuperscript{11-16} However, the studies on how to quantify the phenotype and molecular changes of erythrocytes along with the extracellular environment pH value have never been reported.

Confocal laser tweezers Raman system (LTRS) composed by Raman spectroscopy and laser optical tweezers is used to manipulate the micro object and acquire its Raman spectra simultaneously within a few seconds.\textsuperscript{17-19} Raman spectrum, a sensitive fingerprint, reflects the molecular structures and conformations of biomedical samples, and any physical and chemical properties changes of the material are likely to be shown by it; Optical tweezers can capture a particle from a few to a few hundred microns, so most of the biological tissues and live cells suspended in biological media can be firmly captured without contact.\textsuperscript{20-22} Compared with the previous physical and chemical fixed cell, optical tweezers is a non-contact capture approach, which does not affect the normal physiology environments of cell to get the most realist information feedback. Therefore, confocal LTRS is a suitable tool for studying the living erythrocytes and \textit{in vivo} acquiring their real molecular information under the different pH conditions.\textsuperscript{23} Herein, the home-built LTRS is utilized to continuously study the spectral property of single erythrocytes under different pH values, and the line relationship between them is explored. In this way, the living rats’ arterial blood pH value can be probed continuously and non-invasively for the first time.

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2. Methods

2.1 Experiment setup

The experimental instrument used in our study was a home-built confocal LTRS system. The design of the LTRS system was similar to the previous report. A near-infrared laser at 780 nm (15 mW) was spatially filtered and then introduced into an objective (100×, NA=1.25) of an inverted microscope (Nikon, TE-2000) to form a single-beam optical trap. A cell (levitated 10–μm above the quartz plate) was trapped in the focused laser beam and Raman scattering light of the cell was excited by the same laser beam. The Raman scattering light from the trapped cell was collected with the same objective, separated from the Rayleigh scattering at 780 nm by a holographic notch filter, and then spatially filtered with a 100 μm pinhole to eliminate the out-of-focus light from the sample. The spatially filtered scattering light was then focused onto the entrance slit of an imaging spectrometer and recorded by a CCD detector to express in the form of spectrum in the computer. The Raman spectrum was collected from 400 to 2200 cm⁻¹ with a resolution of ~6 cm⁻¹.

2.2 In vitro experiments

Preparation of different pH buffer solutions

The phosphate buffered saline (PBS) powder (purchased from Sigma) 1 g was dissolved in 100 mL deionized water and then repackaged to sterilize in volumetric flask. Bovine serum albumin (BSA, 1 g L⁻¹) used for maintaining the normal shape of erythrocytes was added to sterilization PBS oscillating until completely dissolved. 0.1 mol L⁻¹ NaOH and HCl solution was used to modulate pH to 6.5, 7.0, 7.4, 8.0, 8.5, and 9.0, which was calibrated by a commercial pH meter (Mettler Toledo, S220). The pH 7.4 was considered as the normal physiological environment, so pH 6.5-7.4 was acidic and pH 7.4-9.0 was alkaline environment for erythrocytes.

Erythrocytes extraction

A weighed 18-20 g Speecific-pathogen- Free (SPF) healthy rat (purchased from Guangxi Medical University) tail was cut to get blood to heparin anti-coagulation tube. The sterilized PBS (pH=7.4) was added to dilute blood in the proportion of 100:1. The diluted blood was to centrifuge for 10 minutes at 2000 r min⁻¹ (25°C) to get erythrocytes and then the erythrocytes were sucked out by a pipette to different pH PBS solutions.

Raman measurement

The PBS solution containing erythrocytes was dripped into the quartz sample cell and put on the microscope to acquire the Raman spectra. Optical tweezers was used to capture an erythrocyte (as shown Fig. 1(a)) and its Raman spectrum was acquired in the meantime. 30 erythrocytes Raman spectra were collected in each pH and the spectrum data acquisition time was 15 s. The background spectrum was collected at the same conditions without the erythrocytes in the trap.

2.3 In vivo experiments

Acidosis and alkalosis model building

Nine healthy rats (18-20 g, SPF) were randomly divided into three groups to be fed routinely and given drug by lavage at 5 p.m every day. Acidosis group: Rats received 0.05 g mL⁻¹ sodium bicarbonate solution at a dose of 0.02 mL g⁻¹. Alkalosis group: 0.28 mmol L⁻¹ ammonium chloride solutions were given to rats at a dose of 0.02 mL g⁻¹. Control group: Stroke-physiological saline solution was given to rats at a dose of 0.02 mL g⁻¹. After 15 days, these rats were used to Raman and blood gas analysis experiments.

Raman measurements of erythrocytes in the artery

As shown in Fig. 1(b), the rat was injected with chloral hydrate (0.075 mL g⁻¹) intraperitoneally and placed on the microscope stage with the ear being affixed on the slide that tightly covered on the oil immersion objective of microscope. The fast-flowing blood and tissues of the ear could be clearly observed by either eyepiece (EP) or a video camera (VC) as shown in Fig. 1(c). A clip was used to nip the rat’s ear to slow down the blood velocity, so that we could see erythrocytes through the blood vessel one by one, just as shown in Fig. 1(d). Moving the object stage on the x, y, and z direction to look for a clear and slow-flowing artery, in that the erythrocytes was captured and its Raman spectra were acquired by LTRS. Fig. 1(e) showed the image of the captured erythrocytes in vivo. Ten different erythrocytes Raman spectra were collected in each artery blood vessel and three arteries were selected for Raman collection each rat. Raman data acquisition time was 15 s and the background spectrum was collected at the same vessel without the erythrocytes and blood flowing.

2.4 Data Analysis

As for in vitro experiments, 90 spectra were totally recorded from 30 erythrocytes (three times per cell) in each pH; and for in vivo experiments, ten different erythrocytes were captured and measured in each artery blood vessel (three arteries per rat). These spectra were processed (for smoothing, baseline subtraction, and peak detection) by means of a simple background elimination method developed by our group. The peak intensities of selected Raman lines (e.g., 1616 cm⁻¹ and 1603 cm⁻¹) were extracted from all spectra and exported automatically using a self-made program.
3. Results and Discussions

3.1 Raman spectra of single erythrocytes under different pH environments in vitro

The average Raman spectra of erythrocytes under different pH environments are shown in Fig. 2(a), it can be seen that the Raman shifts and shape of the spectra of erythrocytes under different pH conditions look much the same. However, the spectra intensity changes with the changing pH values, which is also shown clearly in Fig. 2(b). In acidic environment, the entire Raman intensity of single erythrocytes decreases with the decreasing pH because a little acidic conditions increase the membrane permeability of erythrocytes for water, which induces more H₂O entering cells and cell volume expansion. So the shape of erythrocytes changes from normal discoid shape (in the middle of Fig. 2(c)) to globular shape (in the top of Fig. 2(c)) in the acidic environment. And correspondingly, the concentration of biomolecules inside the whole erythrocytes decreases because of the swelling of erythrocytes; the other hand, with the decreasing pH deviating more from the normal physiological condition, the hemoglobin begins to be denatured. Hemoglobin is the main ingredient of erythrocyte, thus most of spectral information originating from single erythrocytes belongs to hemoglobin molecules solely (see Fig. S1). In case of that, hemoglobin denature also could result in the decreasing Raman intensity of single erythrocytes (see Fig. S2). In the alkaline environment, with the increasing pH, H₂O flow out from erythrocytes to induce cell volume shrinking, which is shown in the bottom of Fig. 2(c). In contrast, the concentration of biomolecules inside the whole erythrocyte increases because of water loss. Similarly, with the increasing pH deviating more from the normal condition, the denatured hemoglobin should also induce the decrease of the entire Raman intensity of single erythrocytes. However, the actual Raman intensity of single erythrocytes is higher in pH 8.0 than in pH 7.4 (Fig. 2(a)), possibly because the impact of water loss may surpass the denatured hemoglobin in the pH 8.0, while in the range of pH 8.5-9.0, the spectral enhancement caused by water loss may be not enough to offset the weaken by hemoglobin denature, so the spectra intensity in pH 8.5-9.0 is less than the normal physiological condition. Although some regular changes occur on the general Raman intensity of single erythrocytes with the changing pH, it has not found whether there is a quantitative law only relying on the entire Raman signals of single erythrocytes.

Fig. 2 (a) Raman spectra of erythrocytes under different pH environments (6.5-9.0) in vivo; (b) Raman Intensity of single erythrocytes under different pH environments in vitro; (c) The image of erythrocytes under different pH environments in vitro and the pH from top to bottom are 6.5, 7.4 and 9.0.
3.2. The linear relationship between the Raman intensity ratio
\(R_{1603, 1616} \) of single erythrocytes and the extracellular pH values \textit{in vitro}

Besides the entire Raman intensity changing along with the pH, we also find there exists a regulated phenomenon between the Raman intensity ratio \(R_{1603, 1616} \) of single erythrocytes and the extracellular pH values. The characteristic Raman peaks at 1603 and 1616 cm\(^{-1}\) under different pH environments is drawn and displayed as Fig. 3(a), it can be seen that the \(R_{1603, 1616} \) decreases with the pH increasing in the range of pH 6.5-9.0. Fig. 3(b) clearly shows the changing trend of the \(R_{1603, 1616} \) with the variation of pH. In order to further explore the possible relation between the \(R_{1603, 1616} \) and the extracellular pH values from 6.5 to 9.0, a linear graph is made as the Fig. 3(b) by origin 8.0. The good linearity between them is 0.985, which shows that the \(R_{1603, 1616} \) is able to reflect the extracellular pH value quantitatively. Why the \(R_{1603, 1616} \) and the extracellular pH value have such a relation? What are the characteristic Raman peaks at 1603 and 1616 cm\(^{-1}\) attributing to? Table 1 lists the main bands of Raman spectra of single erythrocytes and their tentative assignments according to the published works besides ours.\(^{27-32}\) In detail, 1603 cm\(^{-1}\) is attributed to the C=C plane bending vibration of the phenylalanine and tyrosine, and 1616 cm\(^{-1}\) is attributed to the C=C stretching vibration of tyrosine and tryptophan. Protein in acid and alkaline environment will hydrolyze: (1) in acidic condition, tryptophan is damaged due to protein hydrolysis, the stronger the acidity and the more serious the tryptophan’s damage is, while other amino acids still remain the same in this case. Therefore, the relative concentrations of tryptophan decreases to induce the corresponding increase of \(R_{1603, 1616} \); (2) in the alkaline condition, the racemization of nearly all the amino acids will occur except for the stable tryptophan. Thus the \(R_{1603, 1616} \) decreases with the increasing extracellular pH value. The plot in Fig. 3(b) indicates the Raman intensity ratio \(R_{1603, 1616} \) displays an excellent linear correlation with the extracellular pH value ranging from (6.5-9.0) and the linear equation is \(R_{1603, 1616} = -0.06057pH + 1.45854 \). Based on that, the extracellular pH value could be quantified according to Raman measurements of single erythrocytes.

3.3. The \textit{in vivo} quantitative Raman-pH sensor

The aforementioned \textit{in vitro} experiments showed that the \(R_{1603, 1616} \) of single erythrocytes can be used as the “indicator” of the extracellular pH value. Therefore, a relevant experimental design aiming to establish a novel \textit{in vivo} quantitative Raman-pH sensor has been proposed. First, acidosis and alkalosis rat models were built by lavage administration and the blood pH of acidosis, alkalosis, normal group measured by blood gas analysis is 7.135±0.052, 7.608±0.045, 7.450±0.013; after that, Raman spectra of single erythrocytes from these rats were acquired by LTRS \textit{in vivo} non-invasively one by one. Raman spectroscopy is a continuous and non-contact technique. Recently, more and more studies including our previous research were utilized Raman spectroscopy for noninvasive and \textit{in vivo} drug screening, disease diagnosis and etc.\(^{24,25,33-35}\) \textit{In vivo} detection allow us get the most authentic and reliable biochemical information under normal physiological conditions, which can be utilized directly as powerful reference for medical science.

![Fig. 3](image)

**Fig. 3** (a) The enlarged panel of the characteristic peaks at 1603 and 1616 cm\(^{-1}\) of single erythrocytes under different pH conditions; (b) The linear graphs plotted by \(R_{1603, 1616} \) with the extracellular pH ranging from 6.5 to 9.0.

<table>
<thead>
<tr>
<th>Peak (cm(^{-1}))</th>
<th>Assignments</th>
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<tbody>
<tr>
<td>755</td>
<td>(\nu) (pyr breathing)</td>
</tr>
<tr>
<td>1123</td>
<td>(\nu(C_7-C_8)) (C-N), (C_7)-methyl stretch</td>
</tr>
<tr>
<td>1210</td>
<td>(\sigma(C_m H)), observed in the spectra of single human RBC</td>
</tr>
<tr>
<td>1450</td>
<td>(\sigma(C=C))</td>
</tr>
<tr>
<td>1549</td>
<td>(\nu(C_9-C_7))</td>
</tr>
<tr>
<td>1603</td>
<td>C=C in plane bending mode of phenylalanine &amp; tyrosine</td>
</tr>
<tr>
<td>1616</td>
<td>C=C stretching mode of tyrosine &amp; tryptophan</td>
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The average Raman spectra of erythrocytes from acidosis, alkalosis and normal rats are shown in Fig. 4(a), as highlighted by gray region, it can be seen that the intensity ratio ($R_{1603, 1616}$) gets smaller and smaller from acidosis to alkalosis. Also as shown in the inset of Fig. 4(a), a clear trend of pH is presented by the bar chart. The enlarged panels of the characteristic Raman peaks at 1603 and 1616 cm$^{-1}$ of single erythrocytes under different pH conditions can be seen in Fig. S3, and (a), (b) and (c) represent 3 replicates. What’s more, the pH “indicator” founded in vitro Raman measurements (as shown in Fig. 3(b)), which represents the linear relationship between pH (6.5-9.0) and $R_{1603, 1616}$ can also be used for measuring pH in vivo based on laser trapping and Raman measurements of single erythrocytes inside artery blood vessel. Fig. 4(b) shows the distribution of the dots (pH, $R_{1603, 1616}$) plotted by in vivo Raman measurements under different blood pH (3 replicates for each pH) on the linear graph plotted by in vitro Raman measurements. It can be seen that the pH-dependent Raman intensity ratio ($R_{1603, 1616}$) provided by in vivo Raman measurements also falls into the linear range. In addition, the pH change (7.0-7.6) in blood of live animal is much mild compared to the working curve for a large pH change (6.5-9.0), so we supplemented an experiment in vitro. It can be seen from Fig. S4 there is still a good linear relationship between the Raman intensity ratio ($R_{1603, 1616}$) of single erythrocytes and the extracellular pH in a smaller pH range. Furthermore, the data acquired in vivo experiments is also accord with this linear relationship. The repeatability of in vivo Raman measurements could be referred to Fig. S5, the standard deviations of acidic, normal and alkaline conditions are all about 0.0017. Generally, it is hard for life to survive when the pH values falling outside the range of 6.5-9.0. In this case, we only studied the Raman scattering properties of erythrocytes within pH 6.5-9.0. To demonstrate the linear relationship between $R_{1603, 1616}$ of single erythrocytes and the extracellular environment pH beyond the scope of range 6.5-9.0, a linear graph is shown in Fig. S6 after the pH range has been extended to 6.0-10.0.

![Fig. 4](a) The average Raman spectra of erythrocytes from acidosis, alkalosis and normal rats. The inset is the bar chart for describing the trend of Raman intensity ratio ($R_{1603, 1616}$) corresponding to three different types of models; (b) the distribution of the dots (pH, $R_{1603, 1616}$) (gray) plotted by in vivo Raman measurements under different blood pH on the linear graph (black) plotted by in vitro Raman measurements.

**Conclusion**

The Raman scattering properties of erythrocytes under different pH environments were studied in vitro by home-built confocal LTRS. The Raman intensity ratio ($R_{1603, 1616}$) of single erythrocytes and the extracellular pH presents a good linear relationship ($R^2=0.985$), which indicated the $R_{1603, 1616}$ of single erythrocytes could be potentially utilized as the extracellular pH “indicator”. The linear model of $R_{1603, 1616}$ of single erythrocytes and the extracellular pH were verified via in vivo Raman measurements based on acidosis and alkalosis rat models and further used to reliably measure the different blood pH in living rat. These results demonstrate an in vivo and quantitative Raman-pH sensor of arterial blood based on laser trapping of erythrocytes, and the possibility of Raman tweezers technique utilizing as a reliable, nondestructive and noninvasive tool for blood cell-related disease diagnosis and drug screening.

We are grateful to the National Natural Science Foundation of China (81471696, 81560300, 21475100 and 41273093); Natural Science Foundation of Hubei Province of China (No. 2014CFA002); Guangxi Science Funds (2014GXNSFAA118362).

**References**


